HHS Public Access

Author manuscript

Adv Protein Chem Struct Biol. Author manuscript; available in PMC 2021 June 15.

Published in final edited form as:

Adv Protein Chem Struct Biol. 2020 ; 120: 45–84. doi:10.1016/bs.apcsb.2019.12.001.

Roles of *Porphyromonas gingivalis* and its Virulence factors in periodontitis

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Abstract

Periodontitis is an infection-driven inflammatory disease, which is characterized by gingival inflammation and bone loss. Periodontitis is associated with various systemic diseases, including cardiovascular, respiratory, musculoskeletal, and reproductive system related abnormalities. Recent theory attributes the pathogenesis of periodontitis to oral microbial dysbiosis, in which *Porphyromonas gingivalis* acts as a critical agent by disrupting host immune homeostasis. Lipopolysaccharide, proteases, fimbriae, and some other virulence factors are among the strategies exploited by *P. gingivalis* to promote the bacterial colonization and facilitate the outgrowth of the surrounding microbial community. Virulence factors promote the coaggregation of *P. gingivalis* with other bacteria and the formation of dental biofilm. These virulence factors also modulate a variety of host immune components and subvert the immune response to evade bacterial clearance or induce an inflammatory environment. In this chapter, our focus is to discuss the virulence factors of periodontal pathogens, especially *P. gingivalis*, and their roles in regulating immune responses during periodontitis progression.

Keywords

P. gingivalis; gingipain; fimbria; LPS; capsule; inflammation; periodontal disease; T cell

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Conflicts of Interest Statement

The authors report no conflicts of interest related to this work.

1. Periodontitis

Periodontitis is one of the most prevalent inflammatory diseases. In US, 47% of adults, with 70% of the adult population aged 65 years and older, are afflicted with periodontitis (Eke et al., 2015; Eke, Dye, Wei, Thornton-Evans, & Genco, 2012; Papapanou, 2012). Worldwide, 538 million people suffer from severe periodontal disease and 276 million of whom lost their teeth. The figures are expected to rise as population grows and ages (Kassebaum et al., 2017). In 1999, the annual expenditure on the therapy of *periodontal* disease was estimated to exceed $14 billion in the USA (Brown, Johns, & Wall, 2002). Apart from being the major factor causing loss of teeth, periodontitis is associated with various systemic diseases like atherosclerosis, diabetes, rheumatoid arthritis and adverse pregnancy outcomes like low birth weight (Khader, Dauod, El-Qaderi, Alkafajei, & Batayha, 2006; Kinane, Stathopoulou, & Papapanou, 2017; Madianos, Bobetsis, & Offenbacher, 2013). Periodontal disease may exacerbate these diseases through increased pro-inflammatory mediators or direct infiltration of periodontal pathogens in the circulation (Khader et al., 2006).

2. Periodontal Pathogens and their virulence factors

Periodontitis is induced by microbial biofilm and caused by host-mediated inflammation, which leads to collateral tissue damage and clinical attachment loss (Hajishengallis, Darveau, & Curtis, 2012). Oral microbiota contains the microorganisms found in the oral cavity, which are estimated to be more than 700 different species with distinct subspecies (Aas, Paster, Stokes, Olsen, & Dewhirst, 2005; Dewhirst et al., 2010). A balance between the resident microbiota and host immune response maintains a state of homeostasis, while the disruption of this balance contributes to oral diseases like periodontal disease (Lamont, Koo, & Hajishengallis, 2018). Periodontitis is one typical disease of biofilm-associated infection (Bergstrom, 2006; Hajishengallis & Lamont, 2012; Socransky, Haffajee, Cugini, Smith, & Kent, 1998). Oral bacterial biofilms are among the first to be discovered in human and have been studied extensively. From the time that oral bacterial biofilm was first discovered by James Leon Williams in 1897 as a mass of bacteria adhering to the tooth surface, various periodontal pathogens, including *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *Tannerella forsythia* (formerly *Bacteroides forsythus*), *Porphyromonas gingivalis*, *Prevotella intermedia* and *Treponema denticola*, have been extensively investigated (Aruni, Dou, Mishra, & Fletcher, 2015). These bacteria and their pathogenic roles in periodontitis have been explained in various models, including red complex, specific and non-specific microorganisms, keystone pathogen, and polymicrobial synergy and dysbiosis models (Hajishengallis & Lamont, 2012).

Red complex model recognizes bacteria including *P. gingivalis, T. Denticola* and *T. Forsythia,* popularly known as the "red complex" in gingival plaques, as the major pathogens that are responsible for periodontitis progression (Socransky et al., 1998). The "red complex model" laid solid foundation for the concept that periodontitis is a multi-microbe disease, and leads to other newly emerged models following the new technology development and new discoveries. Most recently, the etiology of periodontitis has shifted in favor of the theory of polymicrobial synergy and dysbiosis (the 'PSD model') (Hajishengallis, Darveau, et al., 2012; Hajishengallis & Lamont, 2012), which proposes that disease is initiated by a

synergistic polymicrobial community, in which different members fulfill distinct roles that result in a combined effect on the oral microbial dysbiosis and host immune disruption. Dysbiosis or imbalance in the ecosystem of microorganisms further leads to change in the host-microbe crosstalk that causes destructive inflammation, bone loss, and periodontitis (Abusleme et al., 2013).

During the formation of the pathogenic community, 'keystone pathogens' modulate the immune response to impair host immune surveillance and tip the balance from homeostasis to dysbiosis. Keystone pathogens can also promote the pathogenesis through the enhancement of direct interaction among the oral pathogens. Virulence factors are integral molecules expressed by an organism at various stages of its life cycle with capacity to damage the host. Formation of the pro-inflammatory environment and induction of oral microbiome dysbiosis require diverse virulence factors expressed by the bacteria, such as proteolytic enzymes, capsule, lipopolysaccharide, fimbriae, and certain other surface structures/ligands (How, Song, & Chan, 2016)

Our and others' reports showed even in relatively low numbers, *P. gingivalis* can interfere with host immune responses and disrupt the symbiosis between the local oral bacteria (Hajishengallis, 2014). It acts as the keystone pathogen in the dysbiosis of oral microbiome and periodontitis development. In this chapter, we will discuss various periodontal pathogens, but focus on *P. gingivalis* and its virulence factors for their important roles in host immune response modulation, which are critical for the pathogen colonization, oral microbial dysbiosis, and periodontitis pathogenesis.

3. *P. gingivalis* and its virulence factors

P. gingivalis is a Gram-negative, anaerobic, rod-shaped bacteria forming black colonies on blood agar. It was detected in 85.75% subgingival plaque of chronic periodontitis patients (How et al., 2016). Prevalence of *P. gingivalis* is related to the severity of periodontal disease and has been identified by researchers as one of the major causative agents in the periodontitis pathogenesis. Recently, the keystone pathogen theory postulates that even at low abundance, *P. gingivalis* is capable of inducing chronic periodontitis by remodeling the commensal bacterial community to promote a state of dysbiosis, which leads to disease (Olsen, Lambris, & Hajishengallis, 2017). *P. gingivalis* has been widely investigated, partially benefited from the well-characterized genomics of different *P. gingivalis* strains and the accessibility of a great variety of mutants (Hajishengallis, Darveau, et al., 2012; Naito et al., 2008; Nelson et al., 2003). *P. gingivalis* is known to produce a wide array of virulence factors that could cause tissue destruction on their own or act through other mediators to induce inflammation (Hajishengallis, 2014).

P. gingivalis and *Streptococcus gordonii* are able to interact to form communities and subsequent colonization of the dental plaque. *P. gingivalis* benefits from its interaction and coaggregation in the subgingival plaque for its destructive effects on periodontal tissues (Kuboniwa et al., 2017). *P. gingivalis* can efficiently modify the host immune response and create an environment favorable to its own and other pathogens' continued persistence (Hajishengallis & Lambris, 2011). Virulence factors of *P. gingivalis* play important roles in

the coaggregation, biofilm formation, and oral microbial dysbiosis. They can either directly act on other bacteria or induce an optimal environment. One example of direct interaction is that *P. gingivalis* binds to GAPDH and surface proteins on the surface of *S. gordonii*, which ultimately favor the formation of *P. gingivalis* biofilms on the tooth surface (Maeda, Nagata, Yamamoto, et al., 2004).

4. *P. gingivalis* LPS

4.1 Structure of *P. gingivalis* LPS

Lipopolysaccharide (LPS) is a pathogen associated molecular pattern (PAMP) or more recently identified as microbe-associated molecular pattern (MAMP) (Medzhitov, 2001). LPS is also a major component of the cell wall (i.e. outer cell membrane) of Gram-negative bacteria, including periodontal pathogen *P. gingivalis* (R.P. Darveau, Arbabi, Garcia, Bainbridge, & Maier, 2002). LPS was well known for its toxicity and the ability to cause unwanted host inflammation, which gave it the name endotoxin. LPS from *E. coli* was found to be able to facilitate the host inflammatory response by stimulating Toll-like receptor 4 (TLR4) (Lien et al., 2000; Poltorak et al., 1998). *P. gingivalis* LPS, like those of *E. coli* and other Gram-negative bacteria, comprises the outer leaflet of the bacteria and is composed of lipid A, core oligosaccharide that forms the backbone of LPS, and O-specific polysaccharide (Schromm et al., 2000). Acting as the core factor of LPS in the induction of immune responses, Lipid A moiety confers toxicity and is structurally composed of a phosphorylated β (1–6) D-glucosamine disaccharide backbone and multiacyl chains acylated by fatty acids at specific positions on the backbone (Dixon & Darveau, 2005). LPS from different bacterial species are structurally different, depending on the fatty acid acyl chain composition of lipid A. The structure difference in LPS may explain the distinct mechanisms through which the host cells recognize bacterial species.

P. gingivalis LPS plays a strong pathogenic role in periodontal tissues. The virulence properties of *P. gingivalis* LPS is determined by its lipid A component. *P. gingivalis* LPS contains multiple forms of lipid A (R. P. Darveau et al., 2004), the chemical structure of which was determined to be a glucosamine beta-(1–6) disaccharide 1-monophosphate acylated by 3-hydroxy-15-methylhexadecanoic acid and 3-hexadecanoyloxy-15-methylhexadecanoic acid at the 2- and 2’-positions, respectively (Ogawa, 1993). Host cells respond to *P. gingivalis* LPS lipid A and produce inflammatory responses in gingival tissues, thereby creat a favorable environment for pathogens to sustain and ultimately cause periodontal disease progression (Herath et al., 2013).

While the basic chemical composition of *P. gingivalis* LPS is similar to that of *E. coli* endotoxin, *P. gingivalis* lipid A structure exhibits different variations of acylation, tetra-acylation and penta-acylation. As a consequence, *P. gingivalis* LPS activates distinctive signaling pathways and initiates differential immune responses. Based on its molecular weight, *P. gingivalis* LPS with penta-acylated lipid A is named *P. gingivalis* $LPS_{1690,}$ for its molecular weight of 1690 Da. Similarly, *P. gingivalis* LPS with tetra-acylated lipid A is called $LPS_{1435/1449}$ (Curtis et al., 2011). Basic mechanism for the production of distinctive *P. gingivalis* LPS remains controversial, which can be attributed to different *P. gingivalis* strains and environmental factors, such as the hemin levels, phosphate availability, and

incubation temperatures (R.P. Darveau et al., 2002) (Rangarajan, Aduse-Opoku, Paramonov, Hashim, & Curtis, 2017).

4.2 Immune responses triggered by *P. gingivalis* LPS

P. gingivalis LPS-induced TLR-specific immune regulatory roles have been extensively investigated. While *E. coli*-LPS can activate TLR4 but not TLR2 (O. Takeuchi et al., 1999), the TLR activations that triggered by *P. gingivalis* LPS are much more complicated (Coats et al., 2009; R. Liu, Desta, Raptis, Darveau, & Graves, 2008). The leading signaling pathway activated by *P. gingivalis* LPS is controversial. Some reports showed that TLR4 exerts a dominant function, while others demonstrated that TLR2 is the major receptor (P.-L. Wang & Ohura, 2002). Reports proved that TLR2 is required for alveolar bone loss caused by *P. gingivalis* infection in animal models. *P. gingivalis* stimulation led to the up-regulation of TLR2 expression and pro-inflammatory cytokine production *in vitro* (Papadopoulos et al., 2013). TLR2 is also critical for *P. gingivalis* LPS-activated nitric oxide, TNF-α, and IL-6 production by macrophages (Holden et al., 2014). The signaling pathways downstream of TLRs are also controversial. Some reports showed that NF-κB pathway is critical in TLR-mediated pro-inflammatory signaling and the production of cytokines, chemokines, and adhesion molecules (R. P. Darveau et al., 2004). NF-κB pathway is also important in alveolar bone loss. Its activation by LPS from non-periodontal pathogen *E. coli*, through TLR4 is enough to cause periodontitis (Li et al., 2014). On the other hand, other pathways including the mitogen-activated protein kinase (MAPK) pathway are also reported to be involved in inflammation and bone loss (Bainbridge & Darveau, 2001).

Recent discovery revealed that the binding and activation of specific TLRs by LPS are determined by the structure of different types lipid A, which is the core component and effector of the LPS molecule. Even small changes in lipid A structure can influence the immune responses (Dixon & Darveau, 2005). Lipid A heterogeneity in *P. gingivalis* LPS led to significantly different innate immune response and pro-inflammatory cytokine production (Herath et al., 2011). When binding to TLR4, penta-acylated *P. gingivalis* LPS_{1690} is similar to classical hexa-acylated *E. coli* LPS in certain ways (Bozkurt, Hakki, Hakki, Durak, & Kantarci, 2017). For example, both *P. gingivalis* LPS_{1690} and *E. coli* LPS elevated the LBP protein expression in human oral keratinocytes. *P. gingivalis* LPS_{1690} acts differently from traditional *E. coli* LPS in several ways. While *P. gingivalis* LPS_{1690} activated NF-κB and p38/MAPK pathways, *E. coli* LPS regulated through NF-κB, p38/MPAK and JNK signaling pathways (Ding, Wang, Darveau, & Jin, 2013). *P. gingivalis* LPS_{1690} may also bind to TLR2. Through TLR2 activation, *P. gingivalis* LPS_{1690} significantly enhanced the transcription of NF-κB in mouse cementoblasts. Comparing to *P. gingivalis* LPS_{1690}, tetra-acylated $LPS_{1435/1449}$ stimulation relied even less on TLR4, and more on TLR2 (Nemoto, Darveau, Foster, Nogueira-Filho, & Somerman, 2006). Indeed, $LPS_{1435/1449}$ acts as a TLR4 antagonist and TLR2 agonist (Herath et al., 2013; Herath et al., 2011). Stimulation with either *P. gingivalis* LPS_{1690} or $LPS_{1435/1449}$ elevated TLR2 expression on the cell surfaces of hGFs, but $LPS_{1435/1449}$ induced higher TLR2 expression than LPS_{1690}. In contrast, *P. gingivalis* LPS_{1690}, but not $LPS_{1435/1449}$, significantly increased TLR4 and MD-2 expression in hGFs. *P. gingivalis* LPS_{1690} activated mainly through NF-κB, while *P. gingivalis* $LPS_{1435/1449}$ primarily induced the p38/MAPK and ERK1/2 signaling pathways.

The structure of lipid A in LPS also determines its binding to a newly found intracellular LPS receptor, caspase-11. *E. coli* LPS can also bind to mouse caspase-11 (homologue of human caspase-4), induce caspase-11 oligomerization, and activate the caspases (Kayagaki et al., 2013). On the other hand, underacylated lipid IVa and tetra-acylated LPS, such as *Rhodobacter sphaeroides* LPS (LPS-RS), cannot induce caspase-11 oligomerization and activation, despite that they can bind to caspase-11 (Shi et al., 2014). Tetra-acylated *P. gingivalis* lipid A was found to evade activation of the noncanonical inflammasome, which enhanced the intracellular survival of *P. gingivalis* in macrophages. In contrast, *P. gingivalis* LPS_{1690}, which contains TLR4 agonist lipid A, induced noncanonical inflammasome activation and led to the production of IL-1β and deprived the survival capability of *P. gingivalis* in macrophages (Slocum et al., 2014). *P. gingivalis* might exploit its TLR4 antagonistic lipid A to evade host clearance by interfering with the activation of TLR4 and caspase-11. Indeed, the lipid A phosphatase, which is responsible for lipid A alterations, was shown to be critical for *P. gingivalis* colonization and even the formation of oral microbiome, indicating the importance of the lipid structure in the processes (Zenobia et al., 2014).

5. Fimbriae

5.1 Types and compositions of fimbriae

P. gingivalis develops fimbriae, or pili, which are thin filamentous surface appendages that protrude from the outer membrane. *P. gingivalis* fimbriae have been among the focuses of research in periodontal pathogens and periodontitis pathogenesis. Fimbriae enhance the biofilm formation, bacterial motility, bacterial adhesion to host cells, and bacterial invasion into the cells. *P. gingivalis* expresses two forms of fimbriae, the long fimbriae (also called major fimbriae) comprised of FimA subunits, and the short fimbriae (or minor fimbriae) with Mfa1 subunit proteins. Both FimA and Mfa1 are critical for host immune responses and pathogenesis of periodontitis, even though their amino acid composition and antigenic properties are different (Amano, 2010). With optimal staining, fimbriae can be observed under microscopes, while different *P. gingivalis* strains showed variance in surface morphology and capability to adhere to host cells (Zheng, Wu, & Xie, 2011).

Long fimbriae are 5 nm wide and extend up to 3 μm long, with sizes varying from 41 to 49 kDa (Dickinson, Kubiniec, Yoshimura, & Genco, 1988; Handley & Tipler, 1986; Lee, Sojar, Bedi, & Genco, 1991). FimA is the shaft subunit, with accessory proteins *FimC*, *FimD*, and *FimE* as minor components of the long fimbriae (Enersen, Nakano, & Amano, 2013; Watanabe et al., 1996). FimE is required for assembly of FimC and FimD onto the distal tip of FimA fibers (Nishiyama et al., 2007), while FimB has been suggested as fimbria anchors and regulators of fimbrial length (Nagano, Hasegawa, Murakami, Nishiyama, & Yoshimura, 2010). Deficiency in FimB expression was reported to lead to the formation of abnormal longer fimbriae, which easily shed from the surface (Nagano et al., 2010).

FimA proteins vary in sizes and amino terminals. Fimbriae can be classified into six types based on nucleotide sequences of the *fimA* gene (type I, Ib, II, III, IV, and V) (Fujiwara, Morishima, Takahashi, & Hamada, 1993). Strains 381, ATCC 33277, and HG565 are type I strains and abundantly fimbriated that showed significant adhesive capability to host tissues

and cells. Strains A7A1, SA2, BH18/10, OMZ314, and OMZ409 belong to type II, while strain BH6/26 is a type III *P. gingivalis*. Type IV strains, such as W50 and W83, are poorly fimbriated. HNA99 belongs to type V (Enersen, Olsen, Kvalheim, & Caugant, 2008). When tested for the *fimA* genotypes of *P. gingivalis*, type II and I are the most widely distributed ones in plaque samples from periodontitis patients (Enersen et al., 2008; Nagano, Hasegawa, Iijima, Kikuchi, & Mitani, 2018).

Short fimbriae, composed of mainly Mfa1 structural subunit proteins, differ from 60 to 500 nm in their length (Enersen et al., 2013). Similar to long fimbriae, short fimbriae also contain accessory proteins, which are named Mfa2–5 (Hasegawa et al., 2016; Hasegawa et al., 2009). Mfa2 is an anchor subunit as well as an assembly and elongation terminator (Hasegawa et al., 2009). Mfa3 is localized at the distal tip of the short fimbriae, implying its role as a ligand to host cell receptors (Hasegawa et al., 2016; Hasegawa et al., 2013). Interestingly, Mfa3, Mfa4, and Mfa5 work closely together, by which the absence of any one of these three accessory proteins would lead to lack of all three in the mature fimbriae (Hasegawa et al., 2016; Hasegawa et al., 2013).

5.2 Roles of fimbriae in biofilm formation

As we mentioned earlier, fimbriae play important roles in many of the adhesive properties of *P. gingivalis*. *P. gingivalis* fimbriae bind to host tissues and cells through a wide variety of host molecules and oral substrates, which include proline-rich proteins and glycoproteins, statherins, fibrinogen, fibronectin, and lactoferrin (Amano, 2003). Besides their adhesive function to host tissues and cells, fimbriae are also important for the interaction of *P. gingivalis* with other oral bacteria (Lamont & Jenkinson, 2000), which is indispensible of biofilm formation. Biofilm formation is a complex process, during which the early colonizers first attach to the tooth surface, followed by the attachment of later colonizers (Kuboniwa & Lamont, 2010; Whittaker, Klier, & Kolenbrander, 1996). Fimbriae have been reported to mediate the aggregation of *P. gingivalis* with other organisms including *Actinomyces viscosus* (Goulbourne & Ellen, 1991), *T. denticola* (Hashimoto, Ogawa, Asai, Takai, & Ogawa, 2003), *S. gordonii* (Maeda, Nagata, Nonaka, et al., 2004), and *Streptococcus oralis* (Maeda, Nagata, Yamamoto, et al., 2004). The molecules bound by the *P. gingivalis* fimbriae have been under investigation. Some manifested molecules include the dentilisin of *T. denticola* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of *S. gordonii* and *S. oralis* (Hashimoto et al., 2003; Maeda, Nagata, Yamamoto, et al., 2004; Y. Park et al., 2005). Interestingly, *P. gingivalis* long fimbriae can also bind to human GAPDH, which imply one pathway in host cell invasion and induction of host responses (Sojar & Genco, 2005). Aside from the major components, the minor components of long fimbriae are also critical for the adherence capability of *P. gingivalis*. It was reported that the mutants lack of *fimC*-, *fimD*-, and *fimE* were less efficient to bind to GAPDH of *S. oralis* as well as fibronectin and type I collagen (Maeda, Nagata, Yamamoto, et al., 2004; Nishiyama et al., 2007).

On the other hand, the roles and working mechanisms of short fimbriae in biofilm formation are likely different from those of long fimbriae. For instance, *P. gingivalis* short fimbriae can bind to SspA and SspB proteins of *S. gordonii* (Lamont, Hsiao, & Gil, 1994). Ssp proteins

are members of the antigen I/II family of streptococcal surface proteins. Interestingly, while the antigen I/II proteins are highly conserved among the streptococcal species, *P. gingivalis* can only adhere to SspA/B proteins of *S. gordonii*, but not antigen I/II from *S. mutans*. Research showed that the *P. gingivalis* Mfa1 specially recognized the amino acid residues 1167–1250 at the C-terminal region of the SspB protein, which was named SspB adherence region (BAR) (Y. Park et al., 2005).

5.3 Host immune responses induced by fimbriae

Compared with wild type *P. gingivalis*, the mutant lacking FimA induced less severe bone loss in an animal periodontitis model (Malek et al., 1994). In addition to the fimbriae's roles in biofilm formation, their effects on host immune responses have also been well investigated (Enersen et al., 2013; Pathirana, O'Brien-Simpson, & Reynolds, 2010). The fimbriae can be recognized by the receptors on epithelial, endothelial, and immune cells, which leads to the activation of the cells and the production of cytokines and adhesion molecules (Amano, 2003).

Long fimbriae activated NF-κB via TLR2 and induce the production of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-8, and IL-6 (Hajishengallis et al., 2006). CD14 is an essential TLR2 co-receptor for *P. gingivalis* FimA (Eskan, Hajishengallis, & Kinane, 2007). Gingival epithelial cells do not express CD14, therefore respond poorly to *P. gingivalis* FimA. In contrast, monocytes express CD14 and respond to FimA (Eskan et al., 2007). *P. gingivalis* fimbriae can induce differential immune responses in different host cells, which might be a strategy exploited by *P. gingivalis* to optimize its colonization and infection.

Long fimbriae of *P. gingivalis* are also able to exploit complement pathway to subvert the host immune clearance. Our research showed that fimbriae interacted with complement receptor 3 (CR3; CD11b/CD18) in monocytes/macrophages, resulting in extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation and inhibition of LPS-induced IL-12 production. *In vivo* results proved that CR3 blockage enhanced IL-12 production and *P. gingivalis* clearance. Furthermore, CR3 antagonist protected the mice from *P. gingivalis*-induced periodontitis (Hajishengallis, Shakhatreh, Wang, & Liang, 2007). Since IL-12 is involved in oral pathogen clearance, fimbriae activated CR3 signaling cross talk with *P. gingivalis*-activated TLR pathways, and inhibit IL-12 production to promote the survival of *P. gingivalis*. Later, our research illustrated that the activation of CXCR4 with *P. gingivalis* interacted with TLR2 signaling. In this process, binding of long fimbriae to CXCR4 and TLR2 led to cAMP-dependent protein kinase A activation, which inhibited host immune clearance of bacteria (Hajishengallis, Wang, Liang, Triantafilou, & Triantafilou, 2008). Another crosstalk pathway is between CXCR4 and CR3, in which *P. gingivalis* fimbriae utilize CXCR4 to induce PI3K-dependent activation of CR3 (Hajishengallis, McIntosh, Nishiyama, & Yoshimura, 2012). Both CR3 and CXCR4 exploitation require FimA and FimCDE to assemble fully functional long fimbriae (Pierce et al., 2009; Min Wang et al., 2007).

Research on the effect of short fimbriae is relatively limited. Infection of FimA (long fimbriae) knockout mutant, mfa1 (short fimbriae) knockout, and double mutant *P. gingivalis*

in an animal model showed that both long fimbriae and short fimbriae contributed to alveolar bone loss in periodontitis (Umemoto & Hamada, 2003). Short fimbriae, similar to long fimbriae, interacted with TLR2 and CD14 and induced the cytokine production in both human monocyte cell lines and mouse macrophages (Hamada, Watanabe, Arai, Hiramine, & Umemoto, 2002; Hiramine, Watanabe, Hamada, & Umemoto, 2003). In dendritic cells, Mfa1 interacts with DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin), which stimulates the engulfment of *P. gingivalis* by dendritic cell but promotes the evasion of antibacterial autophagy and lysosome fusion, and leads to the intracellular persistence of *P. gingivalis* in myeloid dendritic cells (El-Awady et al., 2015).

6. Gingipain

Gingipain is a family of cysteine proteinase, which is also known as “trypsin-like” enzyme (Bostanci & Belibasakis, 2012). Gingipains include arginine-specific gingipains (Rgp, including RgpA and RgpB), which hydrolyze peptide bonds at the carbonyl groups of arginine residue, and lysine-specific gingipain (Kpg), which cleave polypeptides at the C-terminal after lysine residue. Structures of the gingipains are highly conserved among different *P. gingivalis* strains. Kgp is composed of a catalytic domain that specifically recognize Lys-Xaa peptide bond and an immunoglobulin-like domain, which are followed by a C-terminal that contains hemagglutinin-adhesin domain. RgpA’s structure is similar to Kgp, with an Arg-Xaa recognizing catalytic domain, an immunoglobulin-like domain, and C-terminal hemagglutinin-adhesin domain. On the other hand, RgpB possesses the catalytic domain and immunoglobulin-like domain that similar to RgpA and Kgp, but lacks the COOH-terminal hemagglutinin domain (Guo, Nguyen, & Potempa, 2010). Newly Translated products of gingipain genes are inactive zymogens to avoid the unwanted proteolytic activity inside the cell and require further post-translational processing to produce mature functional gingipains. For example, pro-RgpA and pro-Kgp need to go through proteolysis to liberate the hemagglutinin-adhesin domains. In different *P. gingivalis* strains, the mature gingipains can either be transported and docked on the bacterial outer membrane or released into extracellular environment.

Gingipains are accountable for 85% of the extracellular proteolytic activities of *P. gingivalis* and are important in the pathogenesis of periodontitis (de Diego et al., 2014). They cause dysregulated immune responses and inflammation through their involvement in the activation of the host matrix metalloproteinases, inactivation of immune inhibitors, degradation of immune factors, and cleavage of immune cell receptors (Guo et al., 2010; Imamura, Travis, & Potempa, 2003; J. Potempa, Banbula, & Travis, 2000). Inhibitors of gingipains can effectively suppress the pathogenicity of *P. gingivalis* and are studied for their potential therapeutic functions (Kadowaki et al., 2004).

6.1 Roles of gingipains in biofilm formation

Gingipains enhance the interactions of *P. gingivalis* with other periodontal pathogens, including *T. forsythia*, *T. denticola*, and *A. actinomycetemcomitans*, to facilitate their survival and biofilm formation (Bao et al., 2014; Haraguchi, Miura, Fujise, Hamachi, & Nishimura, 2014; Ito, Ishihara, Shoji, Nakayama, & Okuda, 2010). Gingipains can be

directly involved in coaggregation with other bacteria or adhesion to host tissues and cells. *P. gingivalis* mutants without all three gingipains (*rgpA- rgpB- kpg-)* exhibit no coaggregative activity (N. Abe et al., 2004; Ito et al., 2010). Reports showed that the hemagglutinin-adhesin domains of RgpA and Kgp are responsible for the coaggregation (N. Abe et al., 2004; Kamaguchi et al., 2003), which was proved by the observation that *P. gingivalis* mutants deficient in hemagglutinin domain-bearing gingipains (i.e. RgpA and Kgp) and hemagglutinin hagA genes were deprived of their coaggregative capability as well (N. Abe et al., 2004). Furthermore, gingipains are involved in the fimbria-mediated adherence process through the modification and maturation of pro-fimbrilin into fimbriae. RgpB is vital for the processing and maturation of FimA (Kristoffersen, Solli, Nguyen, & Enersen, 2015), while both RgpA and RgpB are required for the proteolytic processing and polymerization of Mfa1 to produce short fimbriae (Lee et al., 2018). Besides their ability to coaggregate with other bacteria, gingipains participate in *P. gingivalis* adhesion to host cells like gingival epithelial cells and gingival fibroblasts, which also expedite their survival and infection (Andrian, Grenier, & Rouabhia, 2004; Grenier et al., 2003; Sakanaka, Takeuchi, Kuboniwa, & Amano, 2016).

Gingipains can also provide nutrients for the bacteria and help their proliferation. They degrade fibrinogen/fibrin, which contribute to the gingival tissue breakdown, inhibition of blood coagulation, and increased bleeding (Ally et al., 2003). Gingipain Kgp cleaves host heme proteins, such as hemoglobin, haptoglobin, and hemopexin, to provide *P. gingivalis* with heme for its growth (Sroka, Sztukowska, Potempa, Travis, & Genco, 2001).

6.2 Immune regulation by gingipains

Gingipains provide *P. gingivalis* the abilities to evade host immune responses and clearance. Some of the mechanisms involved the degradation of extracellular matrix components, such as collagens, cytokines, immunoglobulins, and complement factors (Curtis, Aduse-Opoku, & Rangarajan, 2001). Gingipains degrade the junctional adhesion molecules of the gingival epithelial cells to break down epithelial barrier and increase the LPS and proteoglycan penetration (H. Takeuchi et al., 2019). Gingipains are also involved in regulating the immune responses and the production of immune mediators in various cells. Defensins are antimicrobial peptides in innate immune processes that play an important role in pathogen clearance. In humans, defensins are classified into α-defensins (human neutrophil peptides) and ß-defensins (hBDs) (Dommisch & Jepsen, 2015). Gingipains are reported to degrade both neutrophil-derived α-defensins and ß-defensins (Carlisle, Srikantha, & Brogden, 2009; Maisetta, Brancatisano, Esin, Campa, & Batoni, 2011), which might protect the bacteria from being eradicated. Gingipains reduce the expression of innate immune receptors CD14 on macrophage cell surface, which results in decreased response to bacterial infection (Wilensky, Tzach-Nahman, Potempa, Shapira, & Nussbaum, 2015). In human monocytes, chemical inhibitor of gingipains reduced the IL-1β production, indicated its participation in the regulation of inflammatory (Hamedi et al., 2009).

Recently, complement pathways regained the interests of dental research in that they are critical for *P. gingivalis* to cause microbial dysbiosis in oral cavity (Hajishengallis et al., 2011). Complement system is composed of many proteins and protein fragments, which

work through a cascade of reactions to (1) generate opsonization factors to promote phagocytosis of microbes and damaged host cells, (2) promote inflammation, and (3) lyse and destroy foreign cells by forming the membrane attack complex (MAC). The system can be activated through classical pathway, lectin pathway, or alternative pathway. All three pathways converge at the central stage of C3 activation. C3 is cleaved by C3 convertase to generate C3b and C3a. C3b can act as an opsonization factor to promote phagocytosis, while C3a is an important anaphylatoxin. More importantly, C3b is required for complement cascade to cleaves C5 into C5b and C5a. C5b works together with C6, C7, C8, and C9 to form membrane attack complex (MAC), which generates a pore on cell membrane and leads to the lysis of foreign cells. The “side products” of this process, anaphylatoxins C3a, C4a, C5a, are important pro-inflammatory factors. Gingipains disrupt the activation of complement system and host-microbe homeostasis in the periodontal tissue, which further cause periodontitis. Gingipains degrade components C3, C4, and C5 to inhibit complement activation and the formation of MAC, therefore inhibit the bacterial clearance (Popadiak, Potempa, Riesbeck, & Blom, 2007; M. Potempa et al., 2008). Degradation of C3 and C3b molecules by gingipains could also contribute to decreased bacterial opsonization to reduce antibody-mediated bacterial clearance (Wingrove et al., 1992). Rpgs are more potent in degrading C3, C4, and C5 than Kpg is (Popadiak et al., 2007; M. Potempa et al., 2008). Disruption of complement pathway by gingipains not only inhibits bacterial clearance, but also elevates inflammation. For example, Rgps cleave complement component C5 molecule and degrade C5b to block the formation of MAC. At the same time, anaphylatoxin C5a is released and result in higher inflammation (Liang et al., 2011; Schenkein, Fletcher, Bodnar, & Macrina, 1995; Wingrove et al., 1992). On the other hand, Kpg, but not Rpg, can cleave the C5a receptor (C5aR) on neutrophils, which might be a mechanism to curb the recruitment and anti-bacterial action of neutrophils (Jagels, Ember, et al., 1996; Jagels, Travis, Potempa, Pike, & Hugli, 1996).

Moreover, complement systems are involved in a network of cross-interactions with other signaling pathways (Ricklin, Hajishengallis, Yang, & Lambris, 2010) (Hajishengallis, Abe, Maekawa, Hajishengallis, & Lambris, 2013). Indeed, *P. gingivalis* fimbriae exploit the crosstalks between complement receptor CR3, chemokine receptor CXCR4, and TLR signaling pathways to evade bacterial clearance (Maekawa et al., 2014). Additional complement-TLR crosstalk has been discovered to act through the C5a receptor (C5aR) and C3a receptor (C3aR) signaling, which involve mitogen-activated protein kinases (MAPKs), leading to extracellular signal-regulated kinase (ERK1/2) and the c-Jun N-terminal kinase (JNK) activation (X. Zhang et al., 2007). The production of C5a from C5 by Rgps facilitates the crosstalk (Schenkein et al., 1995; Wingrove et al., 1992). While the complement–TLR crosstalk might intend to serve as a protective mechanism against bacterial infection for the host, enhanced production of TNF-α, IL-1β and IL-6 could upregulate inflammation and cause a detrimental effect in inflammatory diseases (X. Zhang et al., 2007). C5aR and C3aR can be potential therapeutical targets since reports showed that blockade of C5a receptor (C5aR) and C3a receptor (C3aR) signaling decrease inflammation and ameliorate the periodontitis (T. Abe et al., 2012; Hajishengallis et al., 2011; Ricklin et al., 2010).

7. Capsules of *P. gingivalis*

The bacterial capsule is an outer envelope structure that lies outside bacterial cell. Capsule usually consists of polysaccharides and contains water to keep the bacterial cells from desiccated. It is important for the bacteria to survive in less-than-optimal environment. Capsule also acts as a protective mechanism in that encapsulated bacteria are more resistant to phagocytosis and intracellular killing. There are many bacterial strains that possess capsules, which protect the bacteria from host immune clearance. One well-known example in textbooks is *Streptococcus pneumonia*, whose capsule was verified as important virulence factors in early 20th century, which showed that the encapsulated pneumococci were virulent while the non-encapsulated variants were avirulent (Griffith, 1928).

The capsule of *P. gingivalis* is also known as K-antigen (Bostanci & Belibasakis, 2012; Holt, Kesavalu, Walker, & Genco, 1999; Schifferle, Reddy, Zambon, Genco, & Levine, 1989). The gene loci of *P. gingivalis* capsule have been identified and characterized (Aduse-Opoku et al., 2006). The chemical composition of *P. gingivalis* capsules varies, by which different *P. gingivalis* strains can be categorized into different K-serotypes, while some *P. gingivalis* strains are not encapsulated at all (Laine, Appelmelk, & van Winkelhoff, 1997). For example, *P. gingivalis* strains ATCC 33277 and W83 have been extensively used in our and others' previous work (Chen et al., 2004; Hajishengallis, 2009; Hajishengallis et al., 2011; Hajishengallis et al., 2008; Liang et al., 2011; Nishikawa & Duncan, 2010; Pierce et al., 2009; Popadiak et al., 2007; Scheres & Crielaard, 2013; M. Wang et al., 2010; Min Wang et al., 2007). Although they both show strong capability to regulate host immune responses and induce periodontitis, ATCC 33277 is not encapsulated and W83 possesses K1 serotype polysaccharide capsules (Brunner et al., 2010; Sheets, Potempa, Travis, Casiano, & Fletcher, 2005; Sheets, Potempa, Travis, Fletcher, & Casiano, 2006). Similar to its functions in other bacteria, *P. gingivalis* capsule protects bacteria from the phagocytosis by host leukocytes, while non-encapsulated *P. gingivalis* strains are less resistant to phagocytosis and killing by macrophages and dentritic cells (Laine et al., 1997; Sundqvist, Figdor, Hanstrom, Sorlin, & Sandstrom, 1991). Encapsulated *P. gingivalis* strain showed much higher survival rate than the non-encapsulated strain (A. Singh et al., 2011). Report showed that different serotype of capsules exhibited differential adhesion capabilities. Overall, the adhesion capacity of encapsulated *P. gingivalis* to gingival epithelial cells was lower than the non-encapsulated ones (Dierickx et al., 2003). The roles of capsules in the decreased adhesion ability might contribute to its protective mechanism from phagocytosis by host cells. Capsules of *P. gingivalis* can also bind to other periodotopathogens to facilitate the co-aggregation of the bacteria and biofilm formation. The binding of *P. gingivalis* to other bacteria is K serotype specific. For example, K5 and K6, but not K1 capsules, can bind to *F. nucleatum* PK 1594 (Rosen & Sela, 2006).

Capsule was shown to induce host immune responses. Different *P. gingivalis* capsular serotypes induced differential cytokine expression in dendritic cells (Vernal et al., 2009) and chemokine production in macrophages (d'Empaire, Baer, & Gibson, 2006). In both cases, W83 strain of K1 serotype was most potent in stimulating chemokine and cytokine production (d'Empaire et al., 2006; Vernal et al., 2009). When the role of capsule in a specific strain was tested, a wild-type encapsulated strain W83 exhibited weaker ability to

induce cytokine production in human gingival fibroblasts than its noncapsulated mutant (Brunner et al., 2010). It is possible that the *P. gingivalis* capsule is a weaker immunity inducer than other virulence factors. *P. gingivalis* capsule antagonizes the strong stimuli and promotes bacterial evasion of host immune responses, therefore promotes bacterial survival in hosts.

Another report demonstrated that encapsulated strain W50 was more resistant to bactericidal effect of antimicrobial peptide, such as defensin HBD3, than its nonencapsulated mutant (Igboin, Tordoff, Moeschberger, Griffen, & Leys, 2011).

In a mouse abscess model, the encapsulated *P. gingivalis* exhibited increased virulence than nonencapsulated strains in forming abscesses. The encapsulated strains caused invasive and spreading abscesses, while nonencapsulated strains caused non-invasive and localized abscesses (Katz, Ward, & Michalek, 1996; Laine & van Winkelhoff, 1998)(A. Singh et al., 2011). Furthermore, when animals were subcutaneously injected with non-encapsulated *P. gingivalis* strain 33277 or strain A7A1–28 of K3 serotype, *P. gingivalis* 33277-challenged animals produced lower levels of serum immunoglobulin G (IgG) and salivary IgA than A7A1-28-challenged ones, implying that capsules may help to induce adaptive immunity and antibody production (Katz et al., 1996). In summary, various reports proved that capsule affected the virulence of *P. gingivalis*. These results showed that the observed differential virulence among the different *P. gingivalis* strains could be at least partially attributed to the inequality in capsular structure and function, while the detailed mechanism of capsule's involvement in the responses needs further investigation and clarification.

8. T cell Immunity induced by *P. gingivalis* and its virulence factors

Bacteria and their virulence factors play important roles in the pathogen interactions and inflammation. Dysregulated host factors like innate immune cells, cytokines, and other soluble factors, which are affected by the virulence factors, caused periodontitis progression. T cells are involved in various inflammatory diseases and are critical in host immune responses. Almost a half-century ago, Ivanyi and Lehner first reported that oral bacteria stimulated lymphocyte activation in mild periodontal disease patients, while this activation was suppressed in severe periodontitis patients (Ivanyi & Lehner, 1970). Later reports showed that T cells from periodontitis patients exhibited a reduced ability to proliferate, suggesting an impaired T cell response during periodontitis (Cole, Seymour, & Powell, 1987). The suppression of T cell activation can be induced by various periodontal pathogens (Shenker & Datar, 1995; Stashenko, Resmini, Haffajee, & Socransky, 1983). Gingipain Rpgs, which down-regulate IL-2 production and attenuate T-cell proliferation, might be used by *P. gingivalis* to evade host adaptive immune system (Khalaf & Bengtsson, 2012). Gingipains can also degrade T cell receptors, such as CD4 and CD8, and down-regulate T cell activation (Hajishengallis et al., 2013; Kitamura, Matono, Aida, Hirofuji, & Maeda, 2002). Interestingly, when tested for mixed lymphocyte reactions (MLR), not all the periodontitis patients showed impaired T cell reactions. For the patients who showed lower MLR responses, the T cells were comprised of lower percentage of naïve T cells. After periodontal therapy in these patients, the MLR responses and naïve T cells went back to normality (Kimura, Fujimoto, & Okada, 1991). While these findings pointed to a protective

function of T cells, recent research demonstrated that the roles of T cell immunity in periodontitis are complicated, partially due to the existence of divergent T cell subsets.

Baker and colleagues found that *P. gingivalis* infection induced bone loss at a lower level in the severe combined immunodeficient (SCID) mice, which lack B and T lymphocytes, than immunocompetent mice (P. J. Baker, Evans, & Roopenian, 1994). Later, Baker et al. further demonstrated that MHC-II deficient mice, which cannot induce $CD4^+$ T cell development and responses, were resistant to alveolar bone loss in a *P. gingivalis*-induced periodontitis model. On the other hand, MHC-I deficient mice that lack CD8+ T cells showed no change in bone loss (Pamela J. Baker et al., 1999). In another report, adoptive transfer of human $CD4^+$ T cells, but not $CD8^+$ T cells, were essential for *A. actinomycetemcomitans* infection-induced alveolar bone destruction in NOD/SCID mice (Teng et al., 2000). After the initial infection of periodontal pathogens, the bacterial antigens will be processed and presented to CD4+ helper T cells by professional antigen-presenting cells, such as dendritic cells, macrophages, and B cells. These findings imply that $CD4^+$ T cells are the major T cell contributors to bone destruction in periodontitis. Therefore, our following discussion has been focused on the $CD4^+$ T-helper cells (Th) and the effects of *P. gingivalis* and its virulence factors on Th cell responses. $CD4^+$ T cells are divided into different subsets Th1, Th2, Th17, Treg, and some other subsets, based on their distinct cytokines and specific transcription factors (Ahlers & Belyakov, 2010). Differentiation and activation of these CD4+ T cell subsets from naïve cells are affected by T cell receptor activation, co-stimulatory factors, and cytokine milieu.

8.1 Th1/Th2 cells

From its establishment, Th1/Th2 paradigm classified the differentiated $CD4^+$ T cells into Th1 and Th2 cells and has prevailed for around two decades (Mosmann, Cherwinski, Bond, Giedlin, & Coffman, 1986). Th1 cells express transcription factors T-bet and STAT-4. They preferably produced cytokines IL-12 and IFN-γ and their differentiation is driven by IL-12. On the other hand, Th2 cells express GATA3 and STAT6, and produce cytokines IL-4, IL-5, IL-6, IL-10, and IL-13 (Jankovic & Feng, 2015). Th1 and Th2 cells respond to different stimulations and are associated with different outcomes in infectious and inflammatory diseases. Th1/Th2 paradigm has been investigated to identify the roles of both cell subsets in periodontitis. By testing the T cell subset cytokine profiles, some studies have shown that the Th1 cells are associated with stable lesions while Th2 cells with progressive lesions (Aoyagi, Sugawara-Aoyagi, Yamazaki, & Hara, 1995; Tokoro, Matsuki, Yamamoto, Suzuki, & Hara, 1997; Wassenaar, Reinhardus, Thepen, Abraham-Inpijn, & Kievits, 1995), suggesting that Th2 cells are associated with more severe disease. Meanwhile, other studies demonstrated that Th1 cells were predominant in the gingival tissues from periodontitis patients and were associated with inflammation and periodontitis severity (Takeichi et al., 2000; Ukai, Mori, Onoyama, & Hara, 2001). Interestingly, there are also papers showed that Th1 and Th2 responses were up-regulated to a similarly higher level during periodontitis (Berglundh, Liljenberg, & Lindhe, 2002).

Th cell differentiation during periodontitis can be at least partially attributed to periodontal pathogens and their virulence factors, with controversy on which specific Th subsets are

preferably promoted. Tests on *P. gingivalis*-responsive T-cell lines from periodontitis and healthy or gingivitis subjects implied that *P. gingivalis* facilitated Th1 differentiation (Gemmell, Grieco, Cullinan, Westerman, & Seymour, 1999). *In vitro* test suggested that fimbriae might induce the IFN-γ production by dendritic cells and provide Th1-biased environment (Ravi Jotwani & Cutler, 2004). On the other hand, there are reports showing that *P. gingivalis* and its LPS induce Th2 responses (Aoyagi et al., 1995; R. Jotwani, Pulendran, Agrawal, & Cutler, 2003). *P. gingivalis* FimA acts through TLR2 and produce IL-10, which suppresses Th1 cell differentiation and function (Gaddis, Maynard, Weaver, Michalek, & Katz, 2013). The capability of *P. gingivalis* to induce Th cell was explored to develop potential vaccine.

In an animal model for periodontitis, IFN-γ- and IL-6-deficient mice were resistant to *P. gingivalis*-induced alveolar bone loss, implying that Th1 and Th2 cells play destructive effects during periodontitis (Pamela J. Baker et al., 1999). Later reports showed that transfer of Th1 or Th1 cytokine IFN-γ caused higher alveolar bone loss in the mice injected with *A. actinomycetemcomitans* or its virulence factors, which indicated the detrimental effects of Th1 cells in periodontitis (Kawai et al., 2000). Interestingly, deficiency in Th1/Th2 cytokines, such as IFN-γ, IL-12p40, IL-4, or IL-10, can lead to more severe alveolar bone loss in aged mice (Alayan, Ivanovski, & Farah, 2007). Since IFN-γ and IL-12 are important in bacterial clearance, and IL-4 and IL-10 are famous for their anti-inflammatory functions, the seemingly contradictory reports might be explained by that the balance between pathogens and different host immune components is required to maintain the periodontal health.

8.2 Th17 cells

Pathogenesis of periodontitis had not been fully explained through the Th1/Th2 paradigm. The discovery of more Th subsets in recent years would help us to further our understanding of the pathogenesis of periodontitis. Among these new subsets are Th17 cells, which express transcription factors RORγt and produce cytokines IL-17 and IL-21. Th17 development and maintenance require cytokines TGF-β, IL-6, IL-21, and IL-23. Th17 is important in protecting against extracellular pathogens and fungi as well as promoting inflammation (O'Connor, Zenewicz, & Flavell, 2010). Recent researches link Th17 to various autoimmune and inflammatory diseases (R. P. Singh et al., 2014; Stadhouders, Lubberts, & Hendriks, 2018; van Bruggen & Ouyang, 2014). Th17 specific cytokine IL-17 and other related cytokines have been found in the gingival tissues or gingival crevicular fluid from periodontitis patients (C. R. Cardoso et al., 2009; Dutzan et al., 2012; Honda et al., 2008; Lester, Bain, Johnson, & Serio, 2007; Moutsopoulos et al., 2012; Ohyama et al., 2009; Vernal et al., 2005). Increased infiltration of Th17 cells in periodontal lesions further proved the association of Th17 with periodontitis (Adibrad et al., 2012; Okui, Aoki, Ito, Honda, & Yamazaki, 2012).

It has been shown that *P. gingivalis* regulate Th17 development and function (Moutsopoulos et al., 2012; Oda, Yoshie, & Yamazaki, 2003). *P. gingivalis*, especially the encapsulated strain W83, induced Th17 in periodontal lesions (Moutsopoulos et al., 2012). Capsules of different serotypes seem to possess differential effects on Th subset polarization. After

priming DCs with the *P. gingivalis* strains of different K-serotypes, strains W83 of serotype K1 and HG184 of K2 induced a Th1/Th17 pattern of immune response, while K3, K4 and K5 strains promoted a Th2 response (Vernal, Diaz-Guerra, Silva, Sanz, & Garcia-Sanz, 2014). Moreover, *P. gingivalis* LPS can promote an environment for Th17 development by inducing the production of IL-17 and IL-23, a cytokine critical for Th17 expansion and maintenance, in human periodontal ligament cells (Y. D. Park et al., 2012). Besides producing pro-inflammatory cytokine IL-17, Th17 lymphocytes also promote bone loss by inducing the expression of RANKL, which is required for osteoclastogenesis (Sato et al., 2006; Stadhouders et al., 2018).

The role of IL-17 in periodontitis is not determined yet and under intense investigation. There are reports showing that IL-17 receptor signaling is protective in periodontitis (Jeffrey J. Yu, Ruddy, Conti, Boonanantanasarn, & Gaffen, 2008; J. J. Yu et al., 2007), and the ones showing that it is detrimental in the disease as well (Eskan et al., 2012). The reason of the inconsistency might be that different animal models were used.

8.3 Treg cells

Regulatory T cell (Treg) represents a special suppressive lymphocyte subset that down-regulates the activation, proliferation, and effector functions of a wide range of immune cells. Tregs specifically express transcription factor Foxp3 and secrete cytokines TGF-β and IL-10. Treg cells also express some cell surface molecules at higher level than naïve helper T cells, such as CD25, CTLA-4, GITR, and etc. Treg cells are critical in the maintenance of host immune homeostasis by controlling the direction and intensity of both adaptive and innate immunity and modulating various host immune responses, including inflammation (Chaudhry & Rudensky, 2013; Shevach, 2018).

Numerous publications on human patients demonstrated that Treg cells were enriched in periodontitis lesions (Cristina Ribeiro Cardoso et al., 2008; Dutzan, Gamonal, Silva, Sanz, & Vernal, 2009; Nakajima et al., 2005). Interestingly, there was also a finding showing reduced $Foxp3^{+}CD25^{+}$ cell number in periodontitis lesions (Ernst et al., 2007). It is still unclear what caused the inconsistency. In a series of reports with animal models for periodontitis, the association of Tregs with periodontitis and the role of Tregs in the disease have been further illustrated. Inhibition of Treg functions with blocking antibody anti-GITR treatment enhanced *A. actinomycetemcomitans*-induced inflammation and bone loss (Garlet et al., 2010), while recruitment of $Foxp3^{+}$ Tregs into gingival tissue inhibited pro-inflammatory cytokine expression and *A. actinomycetemcomitans*-induced alveolar bone resorption in mice (Glowacki et al., 2013). Because all-trans retinoic acid (atRA) promotes the differentiation of Treg cells and sustains Treg stability (Z. M. Liu, Wang, Ma, & Guo Zheng, 2015), atRA was orally administrated to induce an increase of Treg cells and decrease in Th17 cells in cervical lymph nodes, which were accompanied by the inhibition of *P. gingivalis*-induced bone loss (L. Wang, Wang, Jin, Gao, & Lin, 2014).

The roles of Treg-associated molecules have also been tested for their roles in periodontitis. TGF-β and IL-10 are famous anti-inflammatory cytokines and major Treg cytokines. TGF-β and IL-10 were expressed higher in periodontitis tissues (Cristina Ribeiro Cardoso et al., 2008; Nakajima et al., 2005; Yamazaki et al., 1997), while their expression levels were

negatively correlated to the severity of the disease (Dutzan et al., 2009; Dutzan et al., 2012). *P. gingivalis* infection caused more severe alveolar bone loss in IL-10-deficient mice, (Sasaki et al., 2004), and the IL-10 administration suppressed alveolar bone loss, indicating the protective role of IL-10 (Q. Zhang et al., 2014; X. Zhang & Teng, 2006). These findings all support the idea that Tregs play a key role in alleviating periodontitis, while the impairment of Treg function may cause exacerbated tissue damage. Indeed, some reported that during periodontitis, $Foxp3^{+}CD4^{+}$ T cells harvested from periodontitis lesions lost their suppressive function (Okui et al., 2008). Studies have shown that Tregs can convert into IL-17-producing cells in a pro-inflammatory environment (Ayyoub et al., 2009; Koenen et al., 2008; Leveque et al., 2009). Tregs that express RORγt and IL-17 were recently found and believed to play an role in the phenotypic plasticity in T cell lineages, transiting between inflammatory and regulatory function responding to outside stimulation (Nyirenda et al., 2011; Valmori, Raffin, Raimbaud, & Ayyoub, 2010). On the other hand, there are other reports showed that $IL\text{-}17^{+}Foxp3^{+}$ or $ROR\gamma t^{+}Foxp3^{+}$ cells are an independent stable Treg lineage, and are potent in regulating Th17-related diseases (Kluger et al., 2016; B. H. Yang et al., 2016). $IL\text{-}17^{+}Foxp3^{+}$ cells have been detected and seemed to be involved in periodontitis, whose roles are not fully elucidated either (Okui et al., 2012). Since these cells possess both pro-inflammatory and suppressive properties, their roles, protective or destructive, in inflammatory diseases are still unclear (Blatner et al., 2012; Kryczek et al., 2011; Tartar et al., 2010; Voo et al., 2009).

Later *in vitro* and *in vivo* research confirmed the ability of *P. gingivalis* to induce Treg development (Kim et al., 2010; Kobayashi et al., 2011). Interestingly, the effect of *P. gingivalis* on Treg development varies under different conditions. For example, we found that *P. gingivalis* induced fewer Treg cells in pregnant mice than non-pregnant mice. Similarly, infection with *P. gingivalis,* especially the ones with type II FimA, reduced Tregs in atherosclerotic patients, implying that type II FimA might be associated with Treg dysregulation (J. Yang et al., 2014).

Discussion

In this chapter, we have introduced periodontal pathogens and their virulence factors. We focused on *P. gingivalis* because it has been widely investigated and regarded as the keystone pathogen for periodontitis. Despite of its role as a “keystone” pathogen in periodontitis, *P. gingivalis* is not a lone criminal in the progression of periodontitis. Other pathogens and their virulence factors also play critical roles in the pathogenesis of periodontitis, which have been reviewed in other articles (de Andrade, Almeida-da-Silva, & Coutinho-Silva, 2019). *P. gingivalis* uses many methods to cooperate with other pathogens and evade immune clearance in periodontitis. It develops strategies, including their virulence factors, to overcome and exploit host immune pathways to facilitate its own and other pathogens’ colonization and survival. The virulence factors are required for the interactions between *P. gingivalis* and other bacteria. Fimbriae and capsules are able to adhere to the molecules on other bacteria and host tissues and cells to promote the formation of biofilm. The virulence factors can also act on the host cells to inhibit bacterial clearance, promote bacterial invasion, and cause inflammation and tissue damage (Fig. 1). They are critical in the manipulation and exploitation of host immune responses by *P. gingivalis* to lead to dysbiosis

in oral cavity and periodontitis progression. T cells are major components of host immunity. Previous studies confirmed the importance of Th cells in periodontitis pathogenesis. *P. gingivalis* and its virulence factors induce biased Th differentiation and function, while the roles of the T cells in the periodontitis pathogenesis remains controversial.

Great progress has been achieved to understand the complex inter-microbial interactions and host-pathogen interactions during the disease. Previous studies demonstrate that oral bacteria and the host immune system need to maintain a delicate balance. Disruption of the balance would cause dysbiosis in oral cavity, unwanted tissue damage, and diseases. Further exploration on the immune responses induced by the periodontal pathogens and their virulence factors will help us to better understand the pathogenesis of periodontitis and pave the road for its future therapy.

Acknowledgements

S.L. is supported by grants KSEF-3283-RDE-018, KSEF-3835-RDE-020, and NIH DE025388. H.W. is supported by DE026727 from National Institute of Dental and Craniofacial Research, NIH, USA.

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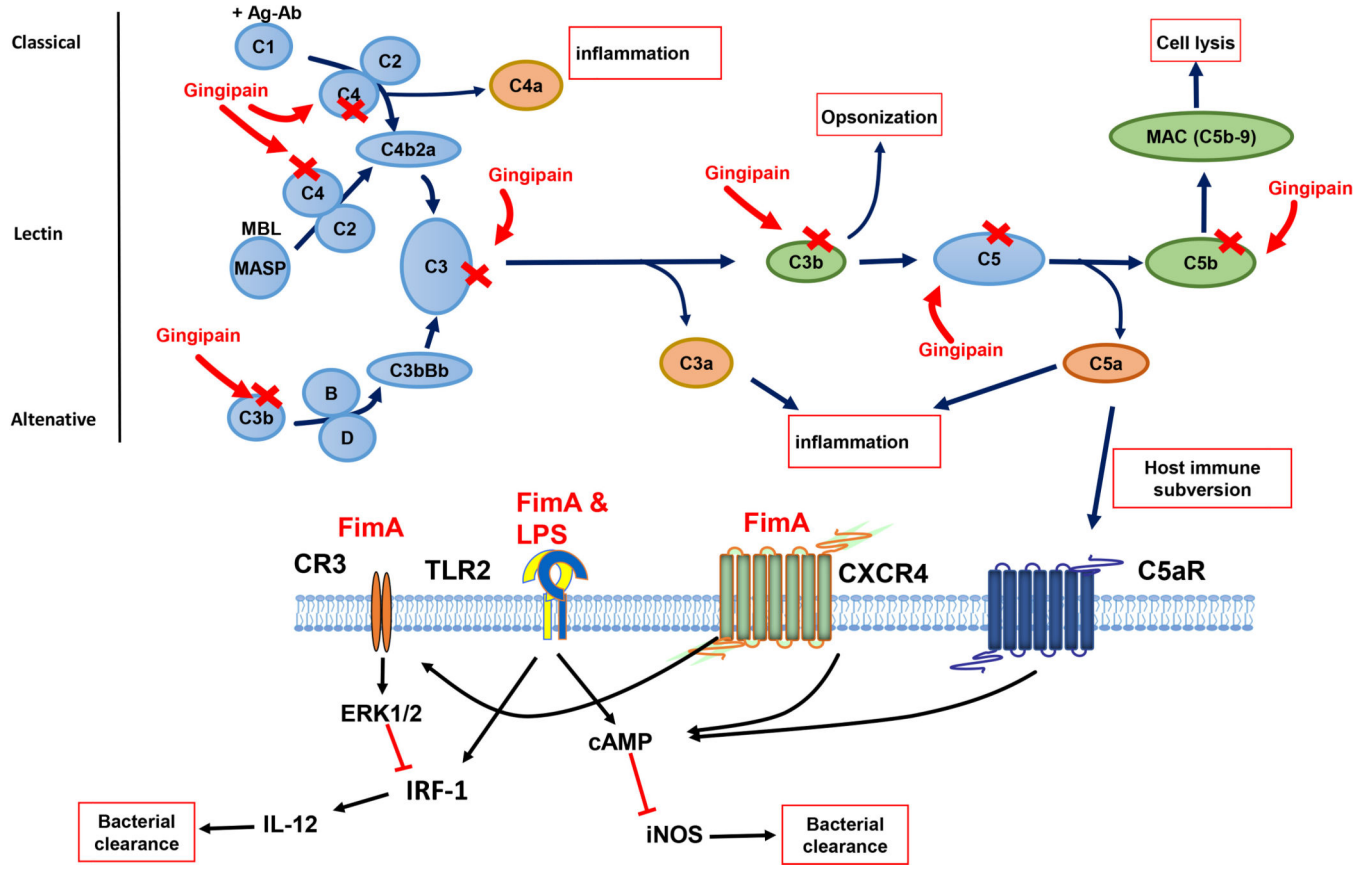

Fig. 1.

P. gingivalis virulence factors subvert immune responses to evade bacterial clearance and promote inflammation. *P. gingivalis* gingipains degrade C3, C4, and C5 to block complement cascade. Besides, anaphylatoxins C3a, C4a, and C5a are generated to promote inflammation. Degradation of C3b prevents opsonization and phagocytosis, and C5b cleavage prevents the formation of membrane attack complex (MAC). Furthermore, C5a crosstalks with TLR2/1 activation and enhances the cAMP responses, which inhibits iNOS production and bacterial killing. Fimbriae promote the crosstalk between CXCR4 and TLR and further enhance cAMP responses. Fimbriae also induce CXCR4-dependent activation of CR3. The interaction between CR3 and *P. gingivalis* requires fimbriae and induces ERK1/2 signaling to down-regulate IL-12 production, which results in impaired bacterial clearance.